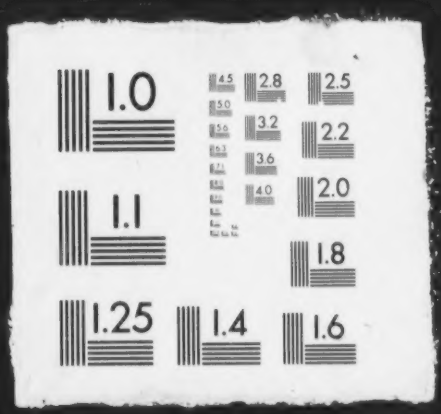
1 OF 1
PB 82
207754



PB82-207754

Comparison of Mutagen Accumulation in 3 Estuarine Species Using the Salmonella/Microsome Activation System

University of West Florida, Pensacola

Prepared for

Environmental Research Lab.
Gulf Breeze, FL

23 Dec 80

U.S. Department of Commerce
National Technical Information Service
NTIS

ERL,GB 0222

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before complet...

1. REPORT NO. EPA-600/J-81-544	2. Journal Article	PB82-207754
4. TITLE AND SUBTITLE: COMPARISON OF MUTAGEN ACCUMULATION IN 3 ESTUARINE SPECIES USING THE SALMONELLA/MICROSOME ACTIVATION SYSTEM		5. REPORT DATE: April 1982 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): T.H. Sparks, J.R. Baylis, and C.W.J. Chang		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Biology and Chemistry Departments University of West Florida Pensacola, FL 32504		10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO.: R806108; 866339
12. SPONSORING AGENCY NAME AND ADDRESS: U.S. Environmental Protection Agency Environmental Research Laboratory Office of Research and Development Gulf Breeze, FL 32561		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE: EPA/600-J

15. SUPPLEMENTARY NOTES: Mutation Research, 85(1981) 133-139

16. ABSTRACT

Three estuarine organisms--oysters (Crassostrea virginica), sea squirts (Mogulla sp.), and shrimp (Peneaus sp.)--were examined for Ames test detectable levels of mutagens. Whole-tissue extracts of these organisms were made and tested using S. typhimurium strains TA98, TA100, TA1535, and TA1538, with and without S9 activation. Positive results were obtained with sea squirts and shrimp extracts. Activation was not necessary to show activity. Toxicity was encountered with oyster extracts. Histidine, a possible source of false positives, was eliminated from shrimp extracts using XAD-2 resin and thick-layer chromatography.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT: Release to public	19. SECURITY CLASS (This Report): unclassified	21. NO. OF PAGES: 6
	20. SECURITY CLASS (This page): uclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA-600/J-81-544
Journal Article

Mutation Research, 85 (1981) 133—139
Elsevier/North-Holland Biomedical Press

COMPARISON OF MUTAGEN ACCUMULATION IN 3 ESTUARINE SPECIES USING THE SALMONELLA/MICROSOME ACTIVATION SYSTEM

TIMOTHY H. SPARKS [1], JOHN R. BAYLIS [1] and CLIFFORD W.J. CHANG [2]

[1] *Biology and* [2] *Chemistry Departments, University of West Florida, Pensacola, FL 32504 (U.S.A.)*

(Received 7 July 1980)
(Revision received 14 October 1980)
(Accepted 23 December 1980)

Summary

3 Estuarine organisms — oysters (*Crassostrea virginica*), sea squirts (Mogulla sp.), and shrimp (Peneaus sp.) — were examined for Ames test detectable levels of mutagens. Whole-tissue extracts of these organisms were made and tested using *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538, with and without S9 activation. Positive results were obtained with sea squirts and shrimp extracts. Activation was not necessary to show activity. Toxicity was encountered with oyster extracts. Histidine, a possible source of false positives, was eliminated from shrimp extracts using XAD-2 resin and thick-layer chromatography.

It is well documented that marine organisms bioaccumulate deleterious environmental compounds (Dunn and Stich, 1976; Lee, 1977; Mix, 1979; Neff et al., 1976; Pancirov and Brown, 1977; Shimkin et al., 1951; Stegeman and Teal, 1973; U.S.D.I., 1964). Most previous studies involve the use of instrumental techniques such as gas—liquid and high-pressure liquid chromatography, atomic absorption, and mass spectrometry. The interphase of a gas—liquid chromatograph with a mass spectrometer, for example, provides a powerful tool for detecting environmental toxicants in minute concentrations. However, the use of these physical tools merely illustrates the sensitivity of these instruments in detecting these compounds.

The use of marine organisms, coupled with bioactivity guided assays, on the other hand, have not been fully employed in monitoring environmental muta-

This work was supported by EPA grants R806108-01 (CWJC) and R806339010 (JRB).

gens/carcinogens. Parry et al. (1976) examined tissue of the mussel *Mytilus edulis* for levels of mutagens using *E. coli* strains in a fluctuation test and *E. coli* and *S. typhimurium* in plate-incorporation tests. Leary and coworkers (1979) have recently employed the Ames test in detecting the presence of naturally occurring mutagenic polyhalogenated hydrocarbons from marine algae.

The intent of this research is to investigate several estuarine species that have been shown to accumulate environmentally undesirable compounds. Problems associated with the possible generation of false positives in the Ames test due to the presence of histidine in shellfish extracts are also examined.

Materials and methods

1. Preparation of extracts

Oysters

Eastern oysters (*Crassostrea virginica*) were collected from an estuarine system surrounded by petroleum industries. Samples were held at 4°C until processed. Oysters were shucked and whole contents removed for use. Shucked oysters, 500 g (wet weight), were homogenized by a Polytron type PT 10/35 tissue homogenizer with 500 ml glass-distilled isopropanol (ACS grade). Separation was accomplished by centrifugation at 5000 rpm for 30 min in a Beckman model J-21C centrifuge at 4°C. After decanting, the marc was then triturated with 500 ml acetone and recentrifuged. The supernatants were evaporated to dryness with a rotary evaporator at 40°C. Test dosages of the extract were prepared in DMSO (Schwartz–Mann spectrometric grade) followed by filter sterilization through a 0.2-μm teflon filter (Gelman No. 66142).

Sea squirts

Sea squirts (Mogulla sp.) were collected from 2 locations. One site (DEV) was located in a bay with several marinas and considerable residential/commercial development. The other collection site (UND) was a bay free of shoreline development. 1 kg (wet weight) of sea squirts was blended with 1.5 l isopropanol and the resultant homogenate was vacuum filtered using a sintered-glass filter. The isopropanol extract was evaporated to dryness under reduced pressure. For testing, aliquots of the dried extract were dissolved in DMSO and filtered through a 0.2-μm teflon filter.

Shrimp

Shrimp (predominantly *Peneaus duorarum*) were purchased from seafood retailers at 3 locations. One location was from the same area as the sampled oysters (sample I, Fig. 1A). The other samples (sample II, sample III) were from retailers located in different areas. For each sample, 1 kg (wet weight) of whole shrimp was blended with 2 l of isopropanol and the resulting homogenate was centrifuged. The supernatant was decanted and evaporated to dryness. Preliminary testing indicated the presence of histidine in the extract. To reduce this problem, 2.0 g of sample I extract was worked up in water and passed through a 0.4 × 16 cm column of Amberlite XAD-2 resin. Prior to use, the resin was washed successively in acetone, methanol, and millipored water.

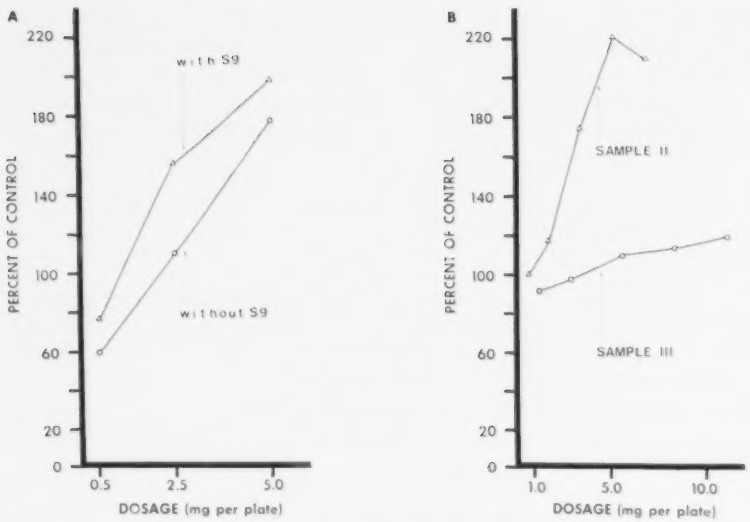


Fig. 1. Dose–response of isopropanol shrimp extracts tested with *S. typhimurium* strain TA98. Revertant counts average of triplicate plates. (A) Sample I tested with (●) and without (○) S9 activation after XAD-2 treatment. (B) Sample II (●), sample III (○), extracts from 2 different sample sites, shown tested with S9 activation.

After loading, the column was washed with 10 ml of water and finally eluted with 40 ml acetone. As before, the extract was evaporated to dryness under reduced pressure and test dosages dissolved in DMSO and filter-sterilized (0.2-μm teflon filters).

In addition, silica-gel thick-layer chromatography was employed to concentrate the extract, and as a means of isolating interfering histidine. 2.0 g of sample II isopropanol shrimp extract were dissolved in 10 ml of methanol. This mixture was reduced to a volume of approximately 2 ml by rotary evaporation and applied on 20 × 20 cm silica gel plates (HF254, Brinkman). The plates were developed in 10% methanol/chloroform. 3 UV-active zones were observed and each was collected and eluted, first with chloroform and then methanol. These were then evaporated to dryness for testing. An identical procedure was performed as a reconstruction experiment with L-histidine (1 mg/ml; Sigma Chemical).

2. Mutation test

The extracts were tested for mutagenicity by the Ames method (Ames et al., 1975). The 5 standard tester strains of *Salmonella typhimurium* – TA98, TA100, TA1535, TA1537, TA1538 – were used in a preliminary screen. Strains were kindly provided by Dr. Bruce N. Ames. Strains showing the greatest activity (TA98, TA100) were selected for further testing. Commercially available Aroclor 1254 Induced Rat S9 (Litton Bionetics) was exclusively used at a concentration of 0.1 ml S9 homogenate per 0.5 ml S9 mix per plate. S9 mix was prepared according to the procedure of Ames et al. (1975). Tests were

performed with and without activation mixture. Spontaneous mutation rate, positive controls, negative control (DMSO), and extracts were tested on triplicate plates and in duplicate experiments in most cases, as suggested by De Serres and Shelby (1979). Cultures were checked periodically for their specific characteristics and were consistent with the data reported by Ames et al. (1975).

Results

Oysters

Oysters, because of their demonstrated ability to concentrate compounds, were examined first. In this investigation, the majority of the oyster extracts showed toxicity in the Ames test. This is evidenced by a decrease in the background lawn, often to the point of no colonies or pinpoint colonies. Table 1 is representative of results from testing oyster extracts. A slight decrease in the number of revertants from the spontaneous rate is shown with the negative control (DMSO). No mutagenicity is indicated in either the isopropanol or acetone extract. The acetone extract shows toxicity without the activation mixture. Toxicity could be due to antimicrobial activity as has been cited by Hillman (1967), and/or the additive effect of DMSO toxicity and antimicrobial activity.

Sea squirts

Table 2 shows data obtained from sea-squirt extracts from both the developed (DEV) and undeveloped (UND) bay systems. The UND extract showed no mutagenicity at a 10-mg dose, whereas the DEV extract with activation showed a 1.7-fold increase over control values, indicating weak mutagenicity.

Shrimp

Testing isopropanol extracts of shrimp after processing through an XAD-2 column gave the results shown in Fig. 1A. At a 5.0-mg dose the extract with S9

TABLE 1

His$^+$ REVERTANTS FROM TESTING OYSTER (*Crassostrea virginica*) EXTRACTS

Strain	Spontaneous revertants [a]		Negative control [b]		Isopropanol oyster extract [c]		Acetone oyster extract [d]	
S. typhimurium	−S9	+S9 [e]	−S9	+S9 [e]	−S9	+S9 [e]	−S9	+S9 [e]
TA98	41	42	15	37	26	38	TOX [f]	27
TA100	162	173	128	186	123	182	TOX [f]	176
TA1538	14	23	11	19	7	25	TOX [f]	24

[a] Revertant counts are the average of duplicate plates.
[b] DMSO at 0.05 ml per plate.
[c] Dosage, 5 mg/0.05 ml/plate.
[d] Dosage, 2 mg/0.05 ml/plate.
[e] S9 level was 0.1 ml S9 fraction/0.5 ml S9 mix/plate.
[f] TOX, indicates toxicity; plates have no background lawn and pinpoint colonies.

TABLE 2

TA100 REVERTANTS (MEAN ± S.E. OF TRIPLICATES) INDUCED BY WHOLE TISSUE, ISOPROPANOL SEA SQUIRT (MOGULLA SP.) EXTRACTS

Source	Mean revertants per plate			
	Without S9 activation		With S9 activation [a]	
	Spontaneous revertants	Extract revertants [b]	Spontaneous revertants	Extract revertants [b]
Undeveloped (UND)	194 ± 8.6	86 ± 3.0	181 ± 11.1	140 ± 6.7
	155 ± 12.3	115 ± 3.7	136 ± 6.2	133 ± 12.4
Developed (DEV)	140 ± 7.0	108 ± 1.5	149 ± 3.5	255 ± 2.0[c]
	232 ± 7.1	141 ± 5.5	252 ± 7.0	337 ± 7.0[c]

[a] S9 level was 0.1 ml S9 fraction/0.5 ml S9 mix/plate.
[b] All testing at 10 mg extract per plate.
[c] Statistically significant at the 5% level using Katz's θ analysis (Katz, 1979).

activation showed a 2-fold increase over the spontaneous reversion number. The extracts without S9 showed a similar dose–response, indicating that the S9 was not necessary to show activity.

Fig. 1B illustrates the dose- response of isopropanol extracts of shrimp from 2 locations. When compared with the control, sample II shows a 2-fold increase

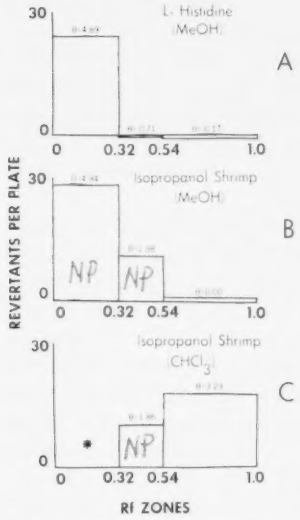


Fig. 2. Reconstruction experiment comparing revertant activity between L-histidine applied onto TLC plates (developed with 10% methanol/chloroform) and isopropanol shrimp extract (sample II), applied similarly. R_f zones were first eluted with chloroform, then methanol. Chloroform gave no extractables from the L-histidine TLC plate (not shown). Spontaneous revertants have been subtracted. Revertant counts average of triplicate plates. Statistics (θ) are performed using Katz's θ analysis (Katz, 1979). Significance at the 5% level where $\theta > 1.64$. Shaded areas represent ninhydrin positive tests. Asterisk (*) indicates no chloroform extractables. The histidine remained at the origin. Significant revertants not attributable to histidine are shown in C ($\theta = 3.23$).

NP

of revertants and weak mutagenicity at the 5.0-mg dose. There is a slight trend for sample III to cause a linear increase in revertants above the control values.

In experiments to concentrate the responsible active compounds, preparative silica-gel thick-layer chromatography was employed. Fig. 2 illustrates R_f zones versus revertant counts. This figure was designed to indicate that the increase of revertants was due to the activity of compounds contained within the R_f zones. L-Histidine used in a reconstruction experiment (Fig. 2A) remained at the origin of the TLC plate, which was developed with 10% methanol/chloroform. The L-histidine was extracted with methanol from the silica-gel band scraped off the plates. The recovered histidine was responsible for a 24 revertant per plate increase over the spontaneous rate, a statistically significant increase (see θ value = 4.89). The isopropanol shrimp extract, when eluted with methanol (Fig. 2B) gave results similar to that of the reconstruction experiment. The methanol-eluted compounds from the first 2 R_f zones of the TLC plates were ninhydrin-positive and produced statistically significant increases in the reversion rates. However, the elution with chloroform (Fig. 2C) produced an active zone that was ninhydrin-negative but which had a statistically significant increase in revertants. The results suggest that the activity cannot be attributed to histidine, which can cause false positive results.

Discussion

In selecting organisms for use in this study, factors such as bioaccumulation abilities, modes of feeding, motility, quantities available, and the likelihood of human consumption were considered. Oysters were investigated because they are known bioaccumulators, sessile, filter feeders, and consumed by man. Neither the isopropanol nor the acetone extracts showed mutagenic activity. There is evidence that toxicity of the oyster extracts was interfering with the mutation test. Methods are being developed to separate the interfering substances from the rest of the extract so the test can be verified. Another approach could be to employ a different mutagen-assay system where antimicrobial activity is not a factor.

Sea squirts are also sessile, easily collected, and have good bioaccumulation abilities. Sea squirts have been shown to accumulate DDT as much as 1000-fold (U.S.D.I., 1964). In this study, a statistically significant degree of mutagenic activity was shown in sea squirts collected from a developed area. This is in contrast to sea squirts obtained in an undeveloped area, which showed no mutagenicity. Although sea squirts do not have the appeal as a food source like oysters and shrimp, their value as a potential monitor should not be overlooked.

Analysis of shrimp extracts indicated shrimp from some sources contain detectable amounts of mutagenic substances. Shrimp obtained from 2 different areas could be distinguished by the Ames test (Fig. 1B). Enhancement of revertant counts with S9 mix may be attributed to histidine in the activation mixture. Activation was not necessary to show activity in shrimp extracts. Shrimp are not ideal biomonitor organisms. Their migration patterns make it difficult to be exact about the sources of their active compounds. However, these results suggest that other less migratory crustaceans could serve as useful biomonitors. The wide use of shrimp as human food may warrant their monitoring for undesirable substances.

Our work has demonstrated a technique for isolating histidine that might otherwise interfere with interpretation of the Salmonella assay. This technique lends itself to other whole-tissue extract testing using the Salmonella assay.

In these studies it has only been possible to compare organisms from different locations. To what extent the responsible mutagenic compounds are natural metabolites and to what extent they are the result of human activities remains to be determined. Isolation of the responsible compounds in the mutagenic extracts is currently under investigation.

For assessment of environmental quality, it is promising that these procedures might be further developed for monitoring mutagens in organisms. Benefits of such development include: (a) establishing priorities for examining point sources of pollution, (b) indications of a kind of pollution not necessarily detected in standard pollution studies, (c) a basis for comparing the level of mutagens present in water vs. tissue concentrations (bioaccumulation), (d) establishing base-line data useful in assessing future perturbations, (e) expanding the knowledge of the occurrence and location of mutagens in the environment.

Acknowledgments

The authors acknowledge the support and interest of Dr. N. Richards (EPA) and the technical assistance of Deda Johansen (UWF).

References

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31, 347—364.

De Serres, F.J., and M.D. Shelby (1979) Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay, Mutation Res., 64, 159—165.

Dunn, B.P., and H.F. Stich (1976) Release of the carcinogen benzo[*a*]pyrene from environmentally contaminated mussels, Bull. Environ. Contam. Toxicol., 15, 398—401.

Hillman, R.E. (1967) Drugs from the Sea, Oceanol. Int., Sept./Oct., 33—37.

Katz, A.J. (1979) Design and analysis of experiments on mutagenicity, II. Assays involving microorganisms, Mutation Res., 64, 61—77.

Leary, J.V., R. Kfir, J.J. Sims and D.W. Fulbright (1979) The mutagenicity of natural products from marine algae, Mutation Res., 68, 301—305.

Lee, R.F. (1977) Accumulation and turnover of petroleum hydrocarbons in marine organisms, in: D.A. Wolfe (Ed.), Fate and Effects of Petroleum Hydrocarbons in Marine Organisms and Ecosystems, Pergamon, Oxford, pp. 60—70.

Mix, M.C. (1979) Chemical carcinogens in bivalve mollusks from Oregon estuaries, EPA 600/3-79-034, U.S. Environmental Protection Agency, 33 pp.

Neff, J.M., B.A. Cox, D. Dixit and J.W. Anderson (1976) Accumulation and release of petroleum-derived aromatic hydrocarbons by four species of marine animals, Mar. Biol., 38, 279—289.

Pancirov, R.J., and R.A. Brown (1977) Polynuclear aromatic hydrocarbons in marine tissue, Environ. Sci. Technol., 2, 989—992.

Parry, J.M., D.J. Tweats and M.A.J. Al-Mossawi (1976) Monitoring the marine environment for mutagens, Nature (London), 264, 538—540.

Shimkin, M.H., B.K. Koe and L. Zechmeister (1951) An instance of the occurrence of carcinogenic substances in certain barnacles, Science, 113, 650.

Stegeman, J.J., and J.M. Teal (1973) Accumulation, release and retention of petroleum hydrocarbons by the oyster *Crassostrea virginica*, Mar. Biol., 22, 37—44.

United States Department of Interior (U.S.D.I.) (1964) Pesticide—Wildlife Studies 1963, A review of fish and wild life service investigations during the calendar year, U.S.D.I. Fish Wildlife Serv. Circ., 199, 12—13.